#572c

Comm.
Dr. Andervont
Dr. Gardner
Dr. Huebner

CARCINOGENESIS

THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.
110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

RECEIVED JUL 2 3 1973

Application for Research Grant
(Use extra pages as needed)

Date 7-17-73

1. Principal Investigator (give title and degrees):
Jay A. Levy, M.D., Assistant Clinical Professor of Medicine, Dept. of Medicine and Research Associate, Cancer Research Institute.

2. Institution & address:
University of California, 3rd & Parnassus Avenues, San Francisco, Calif. 94143

3. Department(s) where research will be done or collaboration provided:
Cancer Research Institute

4. Short title of study:
Development of a model system in vitro for studying carcinomas.

5. ~~Proposed starting date:~~ September 1, 1973

6. Estimated time to complete: 2 years

7. Brief description of specific research aims:

We are endeavoring to obtain a line of mouse epithelial cells which is susceptible to transformation by viruses and chemicals. We shall study the steps involved in epithelial cell transformation and the combined effects of chemicals and viruses on this transformation.

The experience and knowledge gained in the cultivation and study of mouse epithelial cells will be used for research on human epithelial cell transformation.

8. Brief statement of working hypothesis:

Over 80% of human cancers, particularly those of the lung, are carcinomas -- arising from normal epithelial cells. Nevertheless, most of the in vitro systems presently employed to study carcinogenesis use mesenchymal or fibroblast cells and transformation is defined by the production of sarcomas. We have been attempting over the past 2 years to develop a model system in tissue culture for studying epithelial cell transformation. It seems reasonable that epithelial cells will better reflect the malignant changes occurring in vivo, resulting in the production of carcinomas. Moreover, once an epithelial line is established, various contaminants in our atmosphere can be checked for their carcinogenic potential.

9. Details of experimental design and procedures (append extra pages as necessary)

Introduction and Background

It has become evident from data obtained in several laboratories that certain chemicals are capable of inducing carcinomas in vivo (1-3). This induction may lead to the production of specific RNA tumor virus antigens in the tumor (2) and sometimes to a "C" type virus as well (3,4). These observations suggest that chemicals and virus may play a concerted role in the ultimate transformation of some cells.

Similar studies in tissue culture have shown that pretreatment of rodent cells with RNA tumor viruses may increase or help initiate the transforming ability of certain chemicals (5-12). In certain situations the chemical may activate endogenous C-type virus in the tissue culture cells (13,14). In other reports, chemicals transform cells but the possible role of an RNA tumor virus in this transformation has not been examined (15-21). In most cases, however, the cells transformed have been fibroblasts which form sarcomas after inoculation into susceptible hosts. These type tumors do not truly reflect the in vivo experiments leading to carcinoma production. Transformation of rat epithelial cells in tissue culture with subsequent production of carcinomas has been reported (22,23). The possible production of carcinomas by mouse cells transformed in vitro has been suggested (24,25) but not confirmed. One of the main deterents to achieving a reproducible in vitro system for testing carcinoma induction has been the difficulty in obtaining epithelial cell cultures which maintain their differentiation.

Our research is aimed at developing such an in vitro technique. We have chosen the mouse system not only because of the readily available inbred strains but because it is a well defined mammalian cell system and has indigenous and exogenous RNA tumor viruses which have been more carefully and more extensively studied than C-type viruses associated with other animals. Moreover, mouse cells appear to be more readily transformed by chemical than other rodent cells (11). Using mouse cells, then, we can study the events leading up to chemical carcinogenesis as well as the interrelationship of C-type viruses with this transformation event. Our chief objective at first is the establishment of a pure epithelial cell line. Details of our previous attempts were presented in our Annual Report to the Council in 1972. We have learned by these early efforts that the cell morphology in tissue culture is not an accurate method of characterizing cell type. These conclusions are illustrated as well in the results of Sanford, et al. who noted the transformation of epithelial cells to fibroblast-like cells and vice versa in tissue culture (26-28). The best technique

is to transform the cell first and then note the kind of tumor (epithelial or mesenchymal) it produces on inoculation into specific animal hosts.

Our basic procedure has been to obtain epithelial cells from mouse tracheas or skin by standard techniques (29, see Annual Report, 1972) and cultivate those cells which have the morphology of epithelial cells (see Fig. 1-3). Recently we have used a method of floating the skin of newborn mice on top of a trypsin layer (0.25%) overnight. The next day the epithelium can be separated with forceps, and the residual dermis can be removed easily with scalpel and forceps. This technique has given us excellent epithelial cell islands both from mouse skin and mouse tracheas.

In attempts to cultivate these epithelial cells, many different media have been tried including McCoys 5a, RPMI 1640, RPMI 1629, Medium 199 as well as the addition of other growth factors such as vitamin A, sodium pyruvate, and non-essential amino acids. We feel many other potential growth factors should be tried. Attention must be given as well to the percentage of serum in the medium. We have seen that epithelial-like cells, grown in medium containing 10% or 20% fetal calf serum, survive longer than those grown in 2%, but their morphology changes to a more fibroblastic type.

Similar to our experience with NIH Swiss cells, BALB/c mice epithelial-like cells have been easily established in culture when serum levels of 10-20% were employed. These cells show characteristics of contact-inhibition but on transformation they produce only sarcomas in recipient hosts. Similar results have been reported from other laboratories trying to obtain epithelial cell lines (30). We believe strongly, that epithelial cells when encouraged to grow rapidly will lose their differentiation and become fibroblastic.

For this reason we propose to continue those efforts in which factors are added to the media that may maintain cell differentiation and give some stimulation of growth. The tissue culture requirements of primary epithelial cells are obviously different from those defined for standard tissue culture cells and must be specifically determined.

Recently we have isolated a clone of NZB cells from NZB embryos which morphologically resemble epithelial cells (Figs. 4-5). These NZB cells are very sensitive to chemicals and can be transformed with as low as 0.05 µg of 3-methyl cholanthrene (3-MC). A typical focus of cell alteration is illustrated in Fig. 6. The NZB cell line is known to contain a C-type virus but very little infectious virus is produced. Pretreatment with benzanthracene (BA), a relatively non-carcinogenic hydrocarbon, appears to increase the sensitivity of this cell line to chemicals so that detection of small amounts of carcinogen in the atmosphere may be achieved. Although this cell line also produces sarcomas, not carcinomas, in mice (Arnstein, unpublished data), we feel this line holds promise for helping us understand the steps leading up to a transformation event by chemicals. In this sense, the cell line will continue to be useful while we try to establish primarily epithelial cells in culture for subsequent chemical transformation studies.

Methods of Procedure

A. Establishment of epithelial cell lines

1. Tracheas from weanling mice or skin from newborn BALB/c mice will be used since cell inoculation and transplantation experiments are best achieved using an inbred strain. We had originally found that only two 60 mm petri dishes containing epithelial cell islands can be obtained after the trypsinization of 10 mouse tracheas using the technique of differential trypsinization. This method involves exposing the primary plates to 0.25% trypsin for a long enough time period to remove

preferentially scattered fibroblasts without removing the majority of small clusters of epithelial cells which are less sensitive to trypsin and remain attached to the dishes. Islands of epithelial cells which remain are selected for further propagation (Fig. 1-3).

By using a new technique of floating the tracheas or skin on top of a trypsin layer we have been able to double or triple this recovery of epithelial-like cells. The method includes an overnight incubation at 4°C and then removal of the dermis, which separates easily with forceps and scalpel. Many islands of epithelial-like cells can be observed 24 hours after plating. Difficulties, however, arise when we try to establish these cells as continuous lines. Certain growth factors, such as the following will be added to the different media employed.

(a) Vitamin A: This vitamin has been demonstrated as an important factor in maintaining epithelial cell differentiation (31). It encourages epithelial growth in wound healing (32).

(b) Cortisone: Studies by Tomkins and his coworkers indicate that steroids such as dexamethosone are strong inducers of protein production (33). It is also a known inhibitor of fibroblastic growth in vivo (34).

(c) Estrogen

(d) Cyclic AMP

(e) Progesterone: Reboud and Pageaut (35) found that progesterone given to C57 Black mice led to an increase in 20-MC induced cervical carcinomas. The effect of this hormone may be related to a stimulation of cervical epithelial cell growth. It will be interesting to see whether its presence in culture medium will influence epithelial cell growth. The compound may also be effective as a co-carcinogen in vitro as it appears to be in vivo.

(f) Isoproterenol: This compound stimulates DNA synthesis (36).

2. Besides the above method for the cultivation of epithelial cells, a new procedure would be attempted as described below. This method calls for a selected media (medium D) which can be prepared by Grand Island Biologicals (37).

Sequential passage method: Adult tracheas, liver, or skin from mice will be minced and trypsinized (0.25%) for 10 min in a glass flask using a magnetic stirring bar. The tissue fragments will be allowed to settle down and then the cells floating in the supernatant will be collected. A repeat trypsinization of the tissue fragments will be done for an additional 15 minutes and the fragments allowed to settle. The supernatant will then be added to the first collection, and this mixture poured over a nylon gauge (to remove large tissue fragments) into centrifuge tubes. The cells will be centrifuged and gently washed twice; they will then be plated in 75 cm^2 plastic tissue culture flasks. These flasks will be placed in an incubator at 37°C for 20 minutes. After this time period, the medium containing unattached cells will be transferred from each flask to a second flask. The first flask will be refed with medium. The medium from the second flask is then removed after 20 min. and placed in a third flask. This last flask is allowed to remain in the incubator for 2 hours before the medium is withdrawn and discarded. All the flasks are refed with Medium D containing 10% FCS. After 24 hours any fibroblast cells found in the third set will be destroyed by a flamed platinum wire. The first and second flasks are examined for evidence morphologically of epithelial cell growth. Those flasks with predominantly epithelial cells will be saved.

By this method, the fibroblasts are found to attach earliest and by the third passage the majority of the cells should be epithelial-like. Any contaminating fibroblast cells can be removed by the flamed platinum wire. This technique not

only permits recovery of primarily epithelial cells, but also avoids the selection of certain epithelial cells. Such a selection probably results from our other procedures in which small islands of epithelial-like cells are isolated for further propagation.

Results with rat liver cells has shown that an original inoculation of 10^8 cells/flask results in good epithelial cell recovery by the third flask (37). We shall have to experiment with different cell concentrations to determine the optimum for mouse cells.

B. Determination of Cell Type in Tissue Culture.

1. Our most sensitive method for defining a cell type in tissue culture is to transform the tissue culture cells and observe the type of tumor they produce in recipient animals. It is conceivable, however, that epithelial cells lose their differentiation when inoculated into mesenchymal areas of the animal host, and therefore only sarcomas are produced. The same cells might maintain their differentiation if inoculated into animals in areas where epithelial growth normally takes place. For this reason, we are collaborating with Dr. Stuart Yuspa at NIH (see attached letter) and Dr. Arthur Furst in San Francisco. Dr. Yuspa will inoculate our transformed "epithelial-like" cells onto the back of irradiated recipient mice, and note the type of tumors which develop. In preliminary studies with his technique, Dr. Yuspa has been able to transplant skin successfully from various mouse strains (39).

2. Dr. Arthur Furst and his coworkers have demonstrated the production of tumors in the alveoli of mouse lungs after instillation of cell suspensions into the lungs. The alveolar area is an excellent epithelial area for the cultivation of tumors. It is conceivable that transformed epithelial cells will grow into carcinomas more readily in this environment.

3. Histochemical tests to determine the presence or absence of certain cellular enzymes will be conducted. Dr. Clayton Loosli has agreed to help in these studies. Samples of each cell strain will be plated on coverslips at low concentrations to provide colonies of epithelial-like or fibroblast cells. In Dr. Loosli's laboratory in Los Angeles, these colonies will be tested by selected histochemical stains for alkaline phosphatase, glucose-6-phosphate dehydrogenase (G6PD), and lactic dehydrogenase (LDH). With frozen sections of hamster and mouse lung, Dr. Loosli has demonstrated the following differences: Alkaline phosphatase is present in hamster bronchial epithelium and alveolar type II cells, but absent in hamster vascular endothelium and muscle. Glucose-6-phosphate dehydrogenase is only present in hamster bronchial epithelium, whereas LDH may be found in all four areas of the lung.

In mouse lung, on the other hand, alkaline phosphatase is virtually absent from all cells. G6PD is found only in bronchial epithelial cells and LDH is noted in bronchial epithelium and alveolar type II cells but not seen in mouse connective tissue.

We would consequently expect to find G6PD and LDH enzymes in mouse epithelial cells but not in mouse fibroblast cells.

4. Samples of each type of cell line will be examined by electron microscopy (EM). These studies were originally performed by Dr. Ellen Dirksen at our Institute, but must be done subsequently under contract with the EM laboratory at the University. Although the presence of desmosomes is not definitive for epithelial cells (38), their presence and that of other morphologic structures can be suggestive. EM examination has been a helpful method for studying morphologic change in cultured cells.

5. The cells will be plated on coverslips at low concentrations to provide distinct colonies. Cells will be labeled with ^{3}H-TdR to determine their growth characteristics. Epithelial-like colonies usually become labelled peripherally, while fibroblast colonies are equally labelled centrally and peripherally (Crocker and Levy, unpublished observations). This observation suggests that the growth of the epithelial-like cells is controlled by the phenomenon of contact inhibition. This technique may help in the identification of epithelial cell lines.

C. Sensitivity of the cell line to transformation

1. When an epithelial cell line is available, it will be used for studies of carcinogenesis.

a. It will be checked for aryl hydrocarbon hydroxylase (AHH) by the techniques of Nebert, et al. and others (40-42). (This work will be done in collaboration with Dr. Ronald Rasmussen at our Institute and Dr. Richard Kouri in Bethesda).

b. If the enzyme is present, the cells plated at 50% confluency in 60 mm plastic dishes will be treated with varying amounts of hydrocarbons and checked for the induction of transformed foci. Exposure to chemicals will be at first for 10 days and if successful, shorter periods shall be tried.

c. In attempts to look at the kinetics of chemical transformation, the cells will be studied similar to the method used by DiPaoli, et al.(21). Cells will be plated at 5 x 10^2/60 mm dish. After 24 hours they will be exposed to carcinogens dissolved in DMSO. After 48-72 hours or longer, if necessary, they will be washed twice and incubated for an additional 7-8 days. Colonies of transformed cells will be counted.

d. The interrelationship of virus and chemical in the transformation of the cells will be examined, particularly in reference to the initial studies in rodent cells by Freeman, et al., and others (5-12). Similar experiments using non-infected and chronically infected cells will be used. In the previous cases the transformed cells were fibroblasts.

e. The sensitivity of the epithelial cells to transformation by sarcoma viruses will be examined. It would be interesting to note if a sarcoma virus will give a different transformed cell morphology than a chemical, or chemical and leukemia virus together. Ikawa, et al. have suggested that the Kirsten murine sarcoma virus can transform Balb 3T3 cells and give rise to epithelial cell carcinomas (25).

D. Concerted Effect of Virus and Chemical on Transformation of Mouse Tissue Culture Cells.

As stated above, a line of NZB cells has been established which in its early passage maintains a normal epithelial-like morphology. On the addition of small amounts of hydrocarbons, areas of transformation appear which actually stand out from the dish-like "haystacks" (Fig. 6); these foci can be counted easily with the naked eye. We have determined that 3-MC can transform these cells after only 24 hours of treatment with concentrations of 0.5 µg/cc. Recent experiments have suggested that pretreatment of these cells with a non-carcinogenic hydrocarbon may induce high levels of AHH activity so that lower concentrations of hydrocarbons can now be detected by the presence of transformed foci. In this sense, the cell line is made more sensitive to the presence of potential hydrocarbon carcinogens.

Dr. William Benedict in Los Angeles has also used these cells and finds effective transformation with cytosine arabinoside. We propose to continue our studies with the following projects in mind.

1. The cells will be pretreated with non-carcinogenic hydrocarbons and assayed for their possible increased susceptibility to transformation by small concentrations of potential carcinogens. In this manner, the line might be an excellent indicator of potential carcinogens in nature.

2. The carcinogenic potential of various fractions of tobacco smoke and smog air can be tested on the sensitized cells.

3. Since we know cytosine arabinoside induces transformation, we would like to try other antimetabolites such as hydroxyurea, 5-fluorodeoxyuridine and mitomycin (20). These agents work directly on DNA; their mechanism of action may be through chromosome breaks or induction of aneuploidy (44-46).

4. Dr. Sachs and his group have demonstrated a specific chromosome which is present in normal cells but absent after transformation (47,48). They refer to this chromosome as a "suppressor chromosome". In collaboration with Dr. Benedict in Los Angeles, who is examining the possible presence of this phenomenon in the murine system, we will be studying spontaneous transformants in this NZB cell line as well as those cells transformed by chemicals. It will be extremely interesting to note if a uniform absence of a chromosome does determine the malignant transformation of a cell. Similarly, the role various chemicals or antimetabolites may play to encourage the emergence of such a cell can be studied with this sensitive cloned NZB cell line.

5. This cell line, because of its sensitivity to chemicals, lends itself to the study of the timing of chemical transformation. We shall begin by examining the effect of cell synchronization on the degreee of transformation by a chemical. Next, we shall look at specific time periods after cell division to determine when the cell is most susceptible to transformation by a chemical. We shall expose the cell in culture for various time intervals following cell division in attempts to find the ideal time of the transformation event. In a related type of experiment, the cells in suspension will be exposed to chemicals at different concentrations. At various time periods, after incubation (1 h. up to 48 hrs.), aliquots of these cells will be removed, washed twice and plated at low cell density. The quantity of transformed colonies can then be determined. By this method, the length of time required for exposing to a chemical and the effective concentration of a chemical for transformation can be evaluated. Such information may help us understand the point of interaction between the cell's molecular makeup and the chemical.

E. Evaluation of C57 Epithelial-like Cultures

We have recently isolated an epithelial-like cell line from a C57/Bl mouse. This line has a typical "cobblestone" appearance and has the characteristics of contact inhibition (Fig. 7). Several clones of this line are now available. These C57 lines differ from the NZB cell line in that they have no detectable murine leukemia virus. On addition of carcinogenic hydrocarbons no foci of transformation have yet been detected. Toxicity of the chemicals has been demonstrated so we know these cells contain AHH activity.

Over the next year we propose to characterize these cell clones further:

1. We shall infect these cells with murine leukemia virus (MLV) and note if this virus alone can transform the cells after several passages.

2. Cells chronically infected with MLV will be treated with carcinogenic hydrocarbons (particularly 3-methyl cholanthrene) and the combined effect of virus and chemical on transformation will be evaluated. It will be interesting to see if the MLV-infected C57 line acts like the cloned NZB line which has demonstrable endogenous "C" type virus (49-50).

3. We shall repeat the earlier experiments in which the cells were treated with chemical alone. In these new experiments the cells will be treated up to the level of toxicity. Surviving cells will be passed 10 times to determine any carcinogenic effect of the hydrocarbon on the C57 cells.

4. Cell lines will be infected with murine sarcoma virus and any foci formed will be isolated. These transformed cells and any resulting after chemical treatment will be inoculated into the brain of ALS treated NIH Swiss cells [in collaboration with Dr. Arnstein (51)] or into the lungs of recipient C57 mice (in collaboration with Dr. Furst). The type of tumor produced will be evaluated. It is hoped that transformation will produce carcinomas indicating these cells are of epithelial origin.

F. Establishment of Human Epithelial Cell Cultures

A special area for human cell cocultivation has been established for us at the Institute. We propose to begin the cultivation of human skin and tracheal cells using the techniques that are developed in these mouse experiments. These cultures would be available to test carcinogens on human cells. With certain RNA tumor viruses now available which grow in human cells, the possible concerted role of these viruses and chemicals in transformation can be examined. Preliminary experiments with human embryos, tracheas and lungs received from the Department of Pediatrics have indicated that these cells can be propagated.

Significance of the Research

Most human cancers are of epithelial cell origin. For this reason, it is extremely important to develop a model system for studying carcinoma induction. Tissue culture techniques when adapted to this system should offer the most inexpensive and efficient method for an in-depth examination of epithelial cell transformation. The murine system is best adapted to these studies since it is well-defined and its RNA tumor viruses have been well studied and can be detected by a variety of means. No successful cultivation of epithelial cells from mouse trachea and skin has been reported by any laboratory. We feel we have the most experience in these efforts and we would like to continue our attempts using other techniques both in tissue culture and in animals. Until we can develop the methodology for growing pure differentiated epithelial cells in vitro, the precise events leading to epithelial cell transformation in vivo cannot be defined. When a line of epithelial cells has been established, cellular transformation can be easily evaluated by tissue culture procedures which our laboratory has used in other studies of transformation by chemicals and/or viruses.

The work with the NZB and C57 lines will help answer questions about the nature of virus-chemical interactions. It appears that the presence in a cell of a virus (whether infectious or not) is necessary for efficient transformation by chemicals. The timing of the transformation event and the interaction of chemical, virus and host cell DNA can be approached by using these cloned cell lines. Once understood, the results obtained will be helpful for subsequent studies with established murine and human epithelial cell cultures. In summary, the studies presented in this proposal are aimed at answering the important question of how transformation of epithelial cells leading to carcinomas occurs in man.

References

1. Saffiotti, U., Montesano, R., Sellakumar, A.R. and Kaufman, D.G. Respiratory tract carcinogenesis induced in hamsters by different dose levels of benzo-(a)pyrene and ferric oxide. J. Natl. Cancer Inst. 49: 1199-1204, 1972.

2. Whitmire, C.E., Salerno, R.A., Rabstein, L.S., Huebner, R.J. and Turner, H.C. RNA tumor-virus antigen expression in chemically induced tumors. Virus-genome-specified common antigens detected by complement fixation in mouse tumors induced by 3-methylcholanthrene. J. Natl. Cancer Inst. 47: 1255-1265, 1971.

3. Yuspa, S. and Levy, J.A. In vitro cultivation of a chemically induced epidermal carcinoma: Establishment of three cell lines and isolation of murine leukemia virus. J. Natl. Cancer Inst. In Press.

4. Freeman, A.E., Kelloff, G.J., Gilden, R.V., Lane, W.T., Swain, A.P. and Huebner, R.J. Activation and isolation of hamster-specific C-type RNA viruses from tumors induced by cell cultures transformed by chemical carcinogens. Proc. Natl. Acad. Sci. USA 68: 2386-2390, 1971.

5. Freeman, A.E., Price, P.J., Igel, H.J., Young, J.C., Maryok, J.M. and Huebner, R.J. Morphological transformation of rat embryo cells induced by diethyl-nitrosamine and murine leukemia viruses. J. Natl. Cancer Inst. 44: 65-78, 1970.

6. Rhim, J.S., Vass, W., Cho, H.Y. and Huebner, R.J. Malignant transformation induced by 7,12-dimethyl-benz(a)anthracene in rat embryo cells infected with Rauscher leukemia virus. Int. J. Cancer 7: 65-74, 1971.

7. Price, P.J., Freeman, A.E., Lane, W.T., and Huebner, R.J. Morphological transformation of rat embryo cells by the combined action of 3-methylcholanthrene and Rauscher leukemia virus. Nature New Biology 230: 144-146, 1971.

8. Freeman, A.E., Price, P.J., Bryan, R.J., Gordon, R.J., Gilden, R.V., Kelloff, G.J., and Huebner, R.J. Transformation of rat and hamster embryo cells by extracts of city smog. Proc. Natl. Acad. Sci. 68: 445-449, 1971.

9. Rhim, J.S., Creasy, B. and Huebner, R.J. Production of altered cell foci by 3-methylcholanthrene in mouse cell infected with AKR leukemia virus. Proc. Natl. Acad. Sci. USA 68: 2212-2216, 1971.

10. Rhim, J.S., Cho, H.Y., Rabstein, L., Gordon, R.J., Bryan, R.J., Gardner, M.B. and Huebner, R.J. Transformation of mouse cells infected with AKR leukaemia virus induced by smog extracts. Nature 239: 103-107, 1972.

11. Rhim, J.S., Cho, H.Y., Joglekar, M.H. and Huebner, R.J. Comparison of the transforming effect of benzo(a)pyrene in mammalian cell lines in vitro. J. Natl. Cancer Inst. 48: 949-957, 1972.

12. Mishra, N.K. and Ryan, W.L. Effect of 3-methylcholanthrene and dimethylnitrosamine on anchorage dependence of rat fibroblasts chronically infected with Rauscher leukemia virus. Int. J. Cancer 11: 123-130, 1973.

13. Igel, H.J., Huebner, R.J., Turner, H.C., Kotin, P. and Falk, H.L. Mouse leukemia virus activation by chemical carcinogens. Science 166: 1624-1626, 1969.

14. Weinstein, B., Gebert, R., Stadler, U.C., Orenstein, J.M., and Axel, R. Type C

Virus from Cell Cultures of Chemically Induced Rat Hepatomas. Science 178: 1098-1100, 1972.

15. Berwald, Y., and Sachs, L. In Vitro transformation of normal cells to tumor cells by carcinogenic hydrocarbons. J. Natl. Cancer Inst. 35: 641-661, 1965.

16. Mondal, S. and Heidelberger, C. In vitro malignant transformation by methylcholanthrene of the progeny of single cells derived from C3H mouse prostate. Proc. Natl. Acad. Sci. 65: 219-225, 1970.

17. Inui, N. and Takayama, S. Effect of cigarette tar upon tissue culture cells. Neoplastic transformation of hamster lung cells by tobacco tar in tissue culture. British J. of Cancer 25: 574-583, 1971.

18. Katsuta, H. and Takaoka, T. Carcinogenesis in tissue culture. XII. Transformation in culture of rat liver cells with 4-nitroquinoline-1-oxide. Proc. of the 27th General Meeting of Japanese Cancer Assoc., Tokyo, p. 95, 1968.

19. Namba, M., Masuji, H. and Sato, J. Carcinogenesis in tissue culture. IX. Malignant transformation of cultured rat cells treated with 4-nitroquinoline-1 oxide. Japan, J. Exptl. Med., 39: 253-265, 1969.

20. Jones, P.A., Taderera, J.V. and Hawtrey, A.O. Transformation of hamster cells in vitro by 1-β-D arabinofuranosylcytosine, 5-fluorodeoxyuridine and hydroxyurea. Europ. J. Cancer 8: 595-599, 1972.

21. DiPaolo, J.A., Takano, K. and Popescu, N.C. Quantitation of chemically induced neoplastic transformation of BALB/3T3 cloned cell lines. Cancer Res. 32: 2686-2695, 1972.

22. Oshiro, Y., Gerschenson, L.E., and DiPaolo, J.A. Carcinomas from rat liver cells transformed spontaneously in culture. Cancer Res. 32: 877-879, 1972.

23. Williams, G.M., Elliott, J.M. and Weisburger, J.H. Carcinoma after malignant conversion in vitro of epithelial-like cells from rat liver following exposure to chemical carcinogens. Cancer Res. 33: 606-612, 1973.

24. Evans, V.J., Hawkins, N.M., Westfall, B.B. and Earle, W.R. Studies on culture lines derived from mouse liver parenchymatous cells grown in long-term tissue culture. Cancer Res. 18: 261-266, 1958.

25. Ikawa, Y., Gazdar, A.F., and Chopra, H.C. Epithelial features of "nonproducer" BALB/3T3 cells transformed by murine sarcoma virus. J. Natl. Cancer Inst. 49: 1449-1453, 1972.

26. Sanford, K.K., Westfall, B.B., Chu, E.H.Y., Kuff, E., Cavalsky, A.B., Dupree, L.T., Hobbs, G.L. and Carle, W.R. Alterations in morphology, arginase and β-glucuronidase within a close of mouse tumor cells in vitro. J. Natl. Cancer Inst. 26: 1193-1219, 1961.

27. Sanford, K.K., Likely, G.D. and Earle, W.R. The development of variations in transplantability and morphology within a clone of mouse fibroblasts transformed to sarcoma-producing cells in vitro. J. Natl. Cancer Inst. 15: 215-232, 1954.

28. Sanford, K.K., Dunn, T.B., Westfall, B.B., Covalesky, A.B., Dupree, L.T. and Earle, W.R. 'Sarcomatous change and maintenance of differentiation in long-term

cultures of mouse mammary carcinoma. J. Natl. Cancer Inst. 26: 1139-1183, 1961.

29. Kaign, M.E., and Prince, A.M. Production of albumin and other serum proteins by clonal cultures of normal human liver. Proc. Nat. Acad. Sci. USA 68: 2396-2400, 1971.

30. Bomford, R. and Weinstein, I.B. Transformation of a rat epithelial-like cell line by murine sarcoma virus. J. Natl. Cancer Inst. 49: 379-385, 1972.

31. DeLuca, L., Maestri, N., Bonanni, F. and Nelson, D. Maintenance of epithelial cell differentiation: the mode of action of vitamin A. Cancer 30: 1326-1331, 1972.

32. Hunt, T.K., Ehrlich, H.P., Garcia, J.A. and Dunphy, J.E. Effect of Vitamin A on reversing the inhibitory effect of cortisone on healing of open wounds in animals and man. Ann. Surgery 170: 633-641, 1969.

33. Tomkins, G.M., Levinson, B.B., Baxter, J.D. and Dethlefsen, L. Further evidence for post-transcriptional control of inducible tyrosine aminotransferase synthesis in cultured hepatoma cells. Nature New Biol. 239: 9-14, 1972.

34. Stephens, F.O., Hunt, T.K., Jawetz, E., Sonne, M. and Dunphy, J.E. Effect of cortisone and vitamin A on wound infection. Am. J. Surgery 121: 569-571, 1971.

35. Reboud, S. and Pageaut, G. Co-carcinogenic effect of progesterone on 20-methylcholanthrene induced cervical carcinoma in mice. Nature 241: 398, 1973.

36. Baserga, R. Induction of DNA synthesis by a purified chemical compound. Fed. Proc. 29: 1443, 1970.

37. Williams, G.M., Weisburger, E.K. and Weisburger, J.H. Isolation and long-term cell culture of epithelial-like cells from rat liver. Exper. Cell Res. 69: 106-112, 1971.

38. Farquhar, M.G., Palade, G.E. Junctional complexes in various epithelia. J. Cell Biol. 17: 375-412, 1963.

39. Yuspa, S.H., Morgan, D.L., Walker, R.J. and Bates, R.R. The growth of fetal mouse skin in cell culture and transplantation to F_1 mice. J. of Invest. Dermatology 55: 379-389, 1970.

40. Nebert, D.W., Gelboin, H.V. The *in vivo* and *in vitro* induction of aryl hydrocarbon hydroxylase in mammalian cells of different species, tissues, strains and development and hormone states. Arch. Biochem. Biophys. 134: 76-89, 1969.

41. Kouri, R.E., Salerno, R.A. and Whitmire, C.E. Relationships between aryl hydrocarbon hydroxylase inducibility and sensitivity to chemically induced subcutaneous sarcomas in various strains of mice. J. Natl. Cancer Inst. 50: 1973 (In press).

42. Kouri, R.E., Ratrie, H. and Whitmire, C.E. Evidence for a genetic relationship between susceptibility to 3-methylcholanthrene-induced subcutaneous tumors and inducibility of aryl hydrocarbon hydroxylase. J. Natl. Cancer Inst. (In press).

43. Busbee, D.L., Shaw, C.R. and Cantrell, E.T. Aryl hydrocarbon hydroxylase induction in human leukocytes. Science 178: 315-316, 1972.

44. Rosenkranz, H.B. and Levy, J.A. Hydroxyurea: a specific inhibitor of deoxyribonucleic acid synthesis. B.B.A. 96: 181, 1965.

45. Coyle, M.B. and Strauss, B. Cell killing and the accumulation of breaks in the DNA of HEp-2 cells incubated in the presence of hydroxyurea. Cancer Res. 30: 2314, 1970.

46. Benedict, W.F., Gielen, J.E. and Nebert, D.W. Polycyclic hydrocarbon-produced toxicity, transformation and chromosomal aberrations as a function of aryl hydrocarbon hydroxylase activity in cell cultures. Int. J. Cancer 9: 435, 1972.

47. Rabinowitz, Z. and Sachs, L. Control of the reversion of properties in transformed cells. Nature 225: 136-139, 1970.

48. Hitotsumachi, S., Rabinowitz, Z. and Sachs, L. Chromosomal control of revision in transformed cells. Nature 231: 511-514, 1971.

49. Levy, J.A. and Pincus, T. Demonstration of biological activity of a murine leukemia virus of New Zealand black mice. Science 170: 326-327, 1970.

50. Levy, J.A. Studies of C-type viruses from New Zealand Black (NZB) mice. Abstracts of the Annual Meeting of the American Society of Microbiology, 1973, p. 222.

51. Todaro, G.J., Arnstein, P., Parks, W.P., Lennette, E.H. and Huebner, R.J. A type-C virus in human rhabdomyosarcoma cells after inoculation into NIH Swiss mice treated with antithymocyte serum. Proc. Nat. Acad. Sci. USA 70: 859-862, 1973.

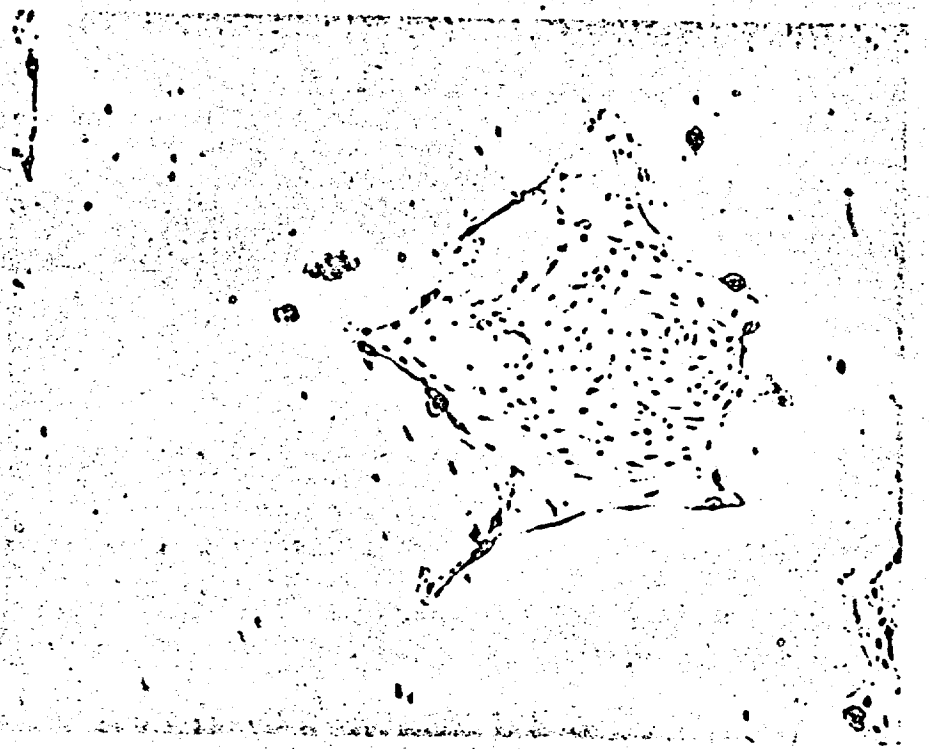

Figure 1

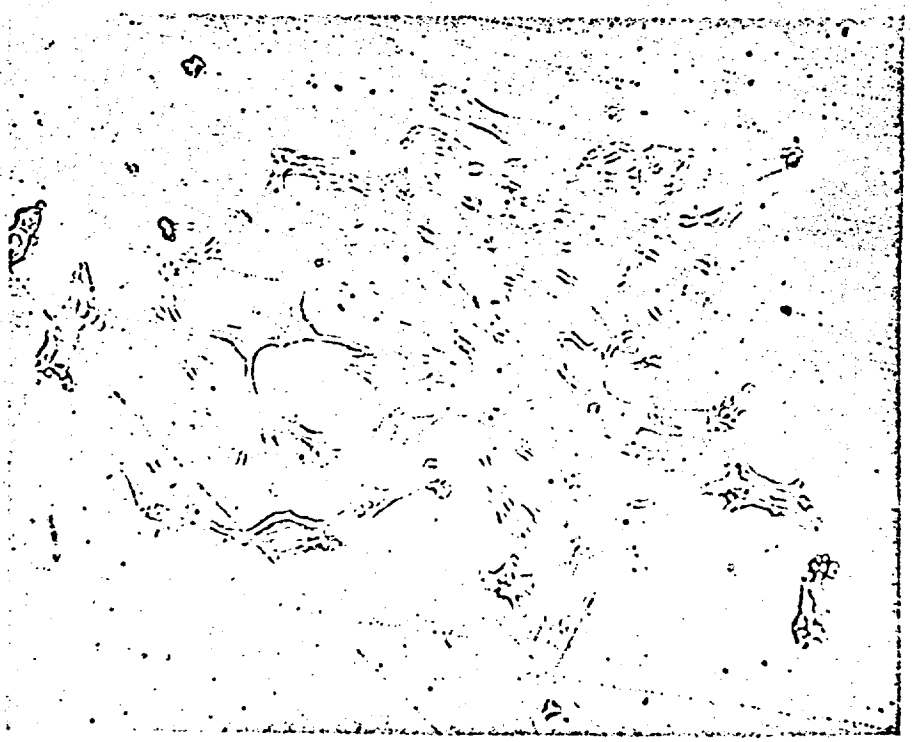

Figure 2

10035404M

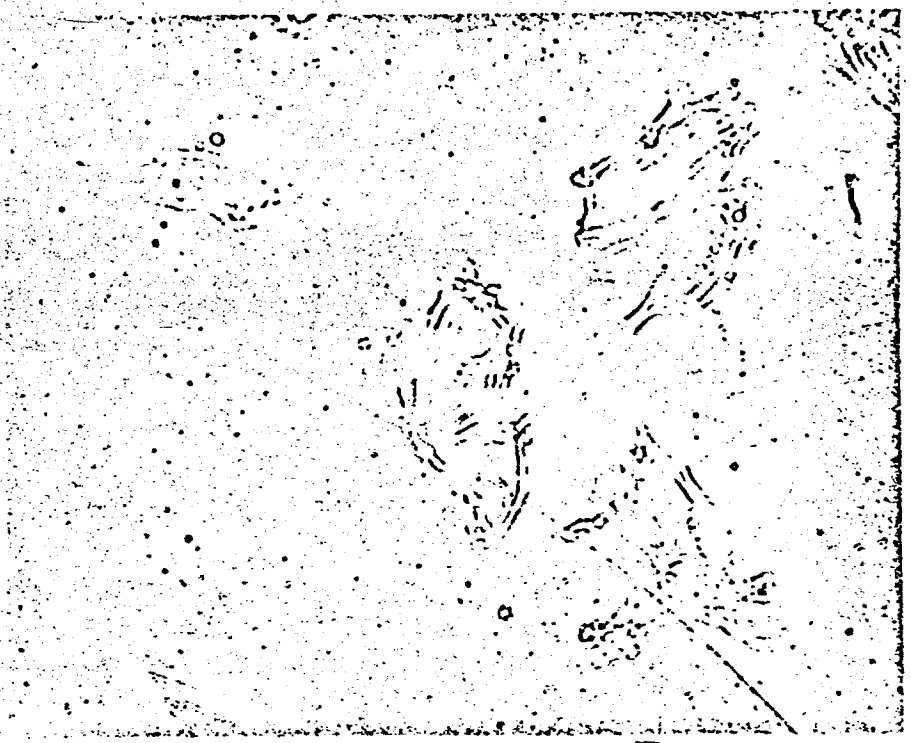

Figure 3

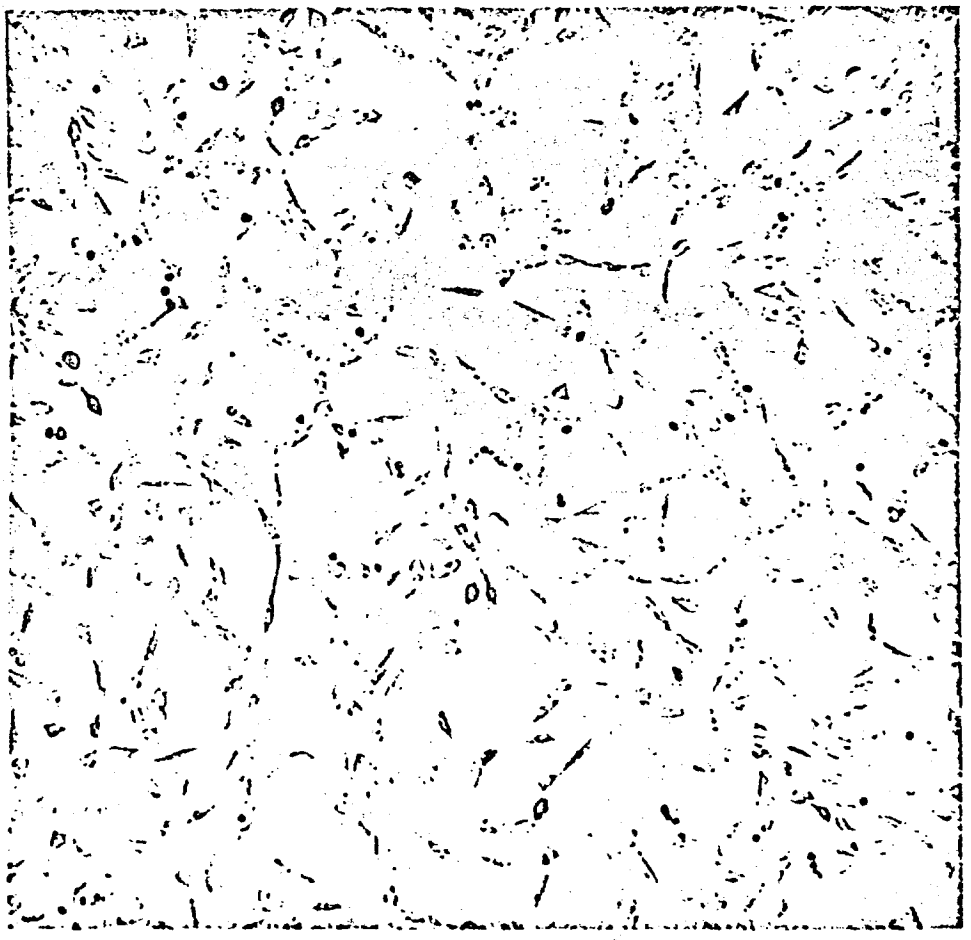

Figure 4

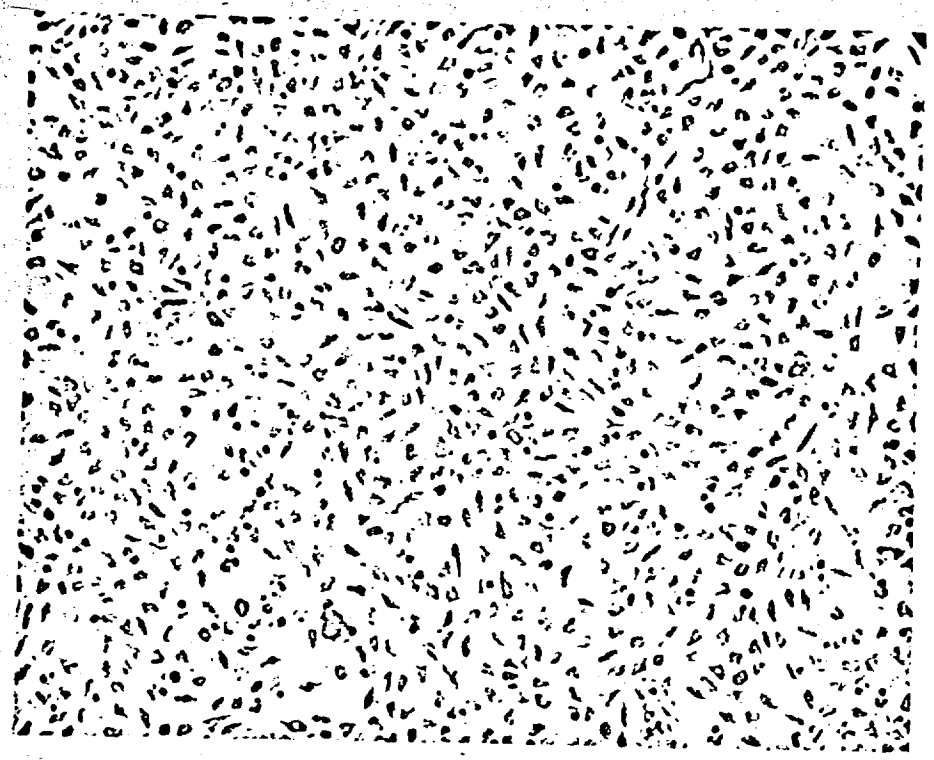

Figure 5

Figure 6

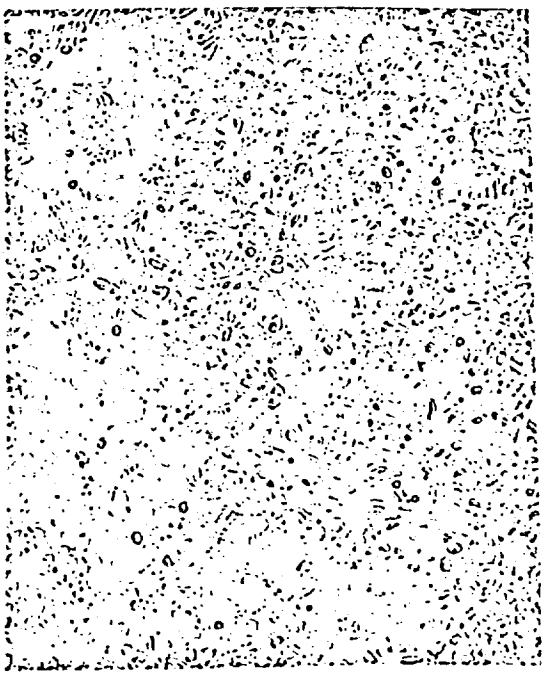

Figure 7

Legend to Figures

Fig. 1-3. Islands of epithelial-like cells recovered after trypsinization of adult BALB/c mouse tracheas .
Fig. 1: x40; Fig. 2: x60; Fig. 3: x60.

Fig. 4. A cloned line of NZB embryo cells established in continuous culture. Note the rectangular epithelioid morphology of the cells. x100.

Fig. 5. The same cloned NZB embryo cell line at confluency. x60.

Fig. 6. An area of cell transformation induced by 3-methylcholanthrene in the cloned NZB embryo cell line.

Fig. 7. A cloned epithelial-like cell line established from a C57/Black mouse. X60.

10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Room 1280 of the Cancer Research Institute, located in the Medical Sciences Building is used for the experimentation with animal cells. This laboratory has approximately 500 square feet with tissue culture units furnished with benches, essential outlets and disposal areas including an autoclave.

Inverted and dissecting microscopes and incubators are in the laboratory. An ultraviolet source for XC testing has been installed and refrigerators and liquid nitrogen tanks for storage of virus and cell lines are present.

A separate area for human cell cultivation has been established in Room 1273-S. It has a Baker 6000 Laminar flow hood, sink, and outlets for an incubator and autoclave. All precautions outlined in the NIH Biological Hazards monograph have been followed (see Appendix).

Additional facilities required:

None

12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

14. First year budget:

A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested) (including 15% fringe benefits)	% time	Amount
Technical		
Siegel, Susan, Staff Res. Assoc. II	100	$ 14,159
Ramirez, Michele, Secretary II	50	4,824
Clark, Vera, Lab Asst. I	50	4,068
Sub-Total for A		$ 23,051*
B. Consumable supplies (by major categories)		
Media and sera		7,500
Animals, purchase and maintenance		1,500
Glassware, pipettes, plastic flasks, petri dishes		2,500
CO_2 tanks and liquid N_2		900
Cells: mouse, rat, human		1,800
Sub-Total for B		14,200
C. Other expenses (itemize)		
EM examinations		2,000
Publication and photography		700
Travel		500
Sub-Total for C		3,200
Running Total of A + B + C		40,451
D. Permanent equipment (itemize)		
Sonicator, ultrasonic system, Biosonik III		950
CO_2 incubator (National)		1,650
Sub-Total for D		2,600
E. Indirect costs (15% of A+B+C)		6,068
Total request		49,119

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	24,204	14,910	3,360	--	6,371	48,845
Year 3						

Justification of the Budget

Salaries:

Our studies with epithelial-like cell cultures have opened up several interesting areas for further research. The techniques for cultivation of epithelial cell clones and characterization of the cell types by animal inoculation and tissue culture testing require the help of a technician and a part-time laboratory worker. The latter's job includes the care of the animals and washing dishware since the Institute does not have a central kitchen.

Susan Siegel has been an integral part of our research team. She has been actively engaged in the cloning and propagation of mouse epithelial cells and the development of sensitive assays for chemical carcinogenesis. Vera Clark, besides keeping us equipped with sterile glassware, has helped in the removal of animal tracheas and skin for epithelial cell cultivation.

The Institute does not provide a secretary; Michele Ramirez has been with us for the past year and we need her for all the budgeting and secretarial tasks that this project entails.

Supplies and Equipment:

Since epithelial cells are very fastidious we must buy already prepared media. These are checked by us beforehand to be certain we have the best lots for maximal cell growth without any toxicity. This media is expensive, but is necessary for optimal results in these studies. Moreover, media, particularly for the sequential passage experiments cited in this proposal, must be specially ordered from Grand Island Biological.

We have not been able to obtain a sonicator for our CF testing. We would like to be able to do our own sonication so that we do not need to request this service from Dr. Huebner's staff. The incubator is needed for maintaining our cell lines.

Others:

The examination of our epithelial cells by electron microscopy has been one interesting method of detecting morphological differences and the presence of non-infectious virus particles. At this time we must pay a set price of $80 for each specimen examined. For $2,000 we can arrange a contract with a group here at the University who will examine all the mouse and tumor specimens we would like over the oncoming year.

16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
None			

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
None			

understood that the investigator and institutional
ers in applying for a grant have read and accept
Council's "Statement of Policy Containing Conditions
Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Jay A. Levy, M.D.

Signature Jay A Levy Date 7-17-73

Telephone 415 (Area Code) 666-4071 (Number) (Extension)

ks payable to

eg[illegible] of the University of California

ing address for checks

ccounting Department - 1550 HSE

niversity of California

an Francisco, California 94143

Responsible officer of institution

Typed Name Mr. Stanley C. Bateman

Title Contracts & Grants Officer

Signature _____ Date _____

Telephone 415 (Area Code) 666-2977 (Number) (Extension)

10035404078

APPENDIX

(Rolf Xerox)

Safety Procedures

1. Baker 6-foot Laminar Flow Hood will be used for experiments with human and cat material.

2. Autoclave will be in close vicinity to inactivate any virus-containing material before general disposal.

3. All virus contaminated glassware will be emersed in Chlorox while awaiting autoclaving.

4. No pipetting by mouth will be permitted in the laboratory.

5. A serum sample will be taken initially from the entire staff and other personnel sharing the rooms. Further samples will be taken and stored every 6 months.

6. All personnel will be supplied with a pair of tennis shoes or comfortable rubber soled shoes for use only in the laboratory. They will be left in the lab at the end of the day. All personnel will wear either a long white coat buttoned to the top or a cloth suit which slips over his day clothes. This will be changed once or twice a week. All personnel supplies will be kept out of the room.

7. No eating or smoking permitted in the lab. Drinking fountains will be the sole source of water for drinking by personnel.

8. No female technician who becomes pregnant will work in proximity to this virus research.

1003544080

#891R1 - NEURATH